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(71) Applicant (for all designated States except US): QUA-DRANT HOLDINGS CAMBRIDGE LIMITED [GB/GB]; Cambridge Research Laboratories, 181A Huntingdon Road, Cambridge CB3 0DJ (GB).

(72) Inventors; and
(75) Inventors/Applicants (for US only) : ROSER, Bruce, Joseph [AU/GB]; The Old Vicarage, Church Lane, Balsham, Cambridgeshire CB1 6OS (GB). COLACO, Camilo [GB/GB]; 107 Foster Road, Trumpington, Cambridgeshire CB2 2JN (GB).

(74) Agent: ABLEWHITE, Alan, James; Marks & Clerk, 57-60 Lincoln's Inn Fields, London WC2A 3LS (GB).

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(54) Title: STABILIZATION OF BIOLOGICAL MACROMOLECULAR SUBSTANCES AND OTHER ORGANIC COMPOUNDS

(57) Abstract

A method of preserving delicate biological substances or organic compounds (a) in a dry state and/or (b) at elevated temperatures and/or (c) under irradiation comprises incorporating in a system containing the said substances or compounds a sugar or a sugar derivative selected from (i) a non-reducing glycoside of a polyhydroxy compound selected from sugar alcohols and other straight chain polyalcohols, or (ii) a non-reducing oligosaccharide selected from raffinose, stachyose and melezitose.

Stabilization of Biological Macromolecular

Substances and other organic compounds

This invention relates to the stabilization of
biological macromolecular substances, in particular
proteins and the like, such as enzymes, antibodies,
fluorescent agents, vaccines etc., under dry and/or hot
and/or irradiated conditions.

Our earlier patent (GB 2,187.171, U.S. 4,891,319)
and applications (PCT/GB89/00047, PCT/GB89/00093)
disclosed and claimed the use of the non-reducing sugar
trehalose to preserve biological compounds while being
dried at ambient temperature. An additional advantage
of that process is that the compounds are not only
stabilized during the drying procedure, but are also
stabilized against long-term storage at relatively high
ambient temperatures. Our earlier application
(EP-A-0415567) diclosed the stabilisation also of low-
molecular weight compounds such as antibiotics,
chelating agents and nucleosides. Other compounds
needing protection include vitamins.

Trehalose is a highly effective agent for this
purpose and, in our subsequent research, has effectively
provided a standard against which any oth r stabilizing
agent must be compared. However, suitably pure

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trehalos is not r adily availabl in large quantities.
There is ther fore a n d for alternative stabilizing
agents. Sugars in general are of limited use. For most
purposes reducing sugars do not provide the degree of
stabilization required, especially during the drying
procedure at a relatively high ambient temperature.
Furthermore, reducing sugars are found to be of very
little help in long-term stabilization and, in some
cases can be highly deleterious. Other apparently
non-reducing sugars, such as sucrose, again have limited
utility and, even if they provide good stabilization
during the drying step, they again provide very inferior
long-term stabilization.

A chance observation that, under unusual
circumstances, trehalose will crystallize on drying and
that biological materials associated with the
crystalline trehalose lacked protection, led to a search
for compounds which could be dried without
crystallizing, which were non-reducing but which were
polyhydroxy compounds capable of replicating the effect
of trehalose. We have now found that one particular
type of carbohydrate material fulfills these
requirements and is extremely effective in stabilizing
biological materials both during the drying step and for
long term storage.

The compounds in question are non-reducing
glycosid s of polyhydroxy compounds s lected from sugar

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alcohols and other straight chain polyalcohols. Other
useful compounds include raffinose, stachyose and
melezitose.

The sugar alcohol glycosides are preferably mono-
glycosides, in particular the compounds obtained by
reduction of disaccharides such as lactose and maltose.
The glycosidic group is preferably a glucoside or a
galactoside and the sugar alcohol is preferably sorbitol
(glucitol).

Particularly preferred compounds are maltitol
(4-O-β-D-glucopyranosyl-D-glucitol), lactitol
(4-O-β-D-galactopyranosyl-D-glucitol) and the two
isomers of palatinit, the sugar alcohol derived from
isomaltulose (palatinose) (6-α-D-glucopyranosyl-
mannitol and 6-α-D-glucopyranosyl-sorbitol).

In comparative tests we have shown that these
compounds are surprisingly very effective in contrast to
other sugar alcohols and other sugars, whether reducing
or not.

An example of the beneficial effect of the compounds
according to this invention is as follows:-

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Mol cular biology techiques which form the basis for
the technology of g n tic ngineering d pend on the use
of highly specific reagents. An important class of such
reagents are the so called restriction enzymes. These
proteins, largely derived from bacterial sources, have
the property of recognising particular sequences of
nucleotides in the DNA chain where they cause scission
of the inter nucleotide bonds and cut the DNA into
precise and reproducible fragments. These enzymes vary
considerably in stability. Some are stable even at
elevated temperature while others are among the most
fragile biological reagents known. As a consequence,
these enzymes are difficult and expensive to store, ship
and handle. Routinely, these reagents are shipped in
small aliquots of a few micrograms packed in solid CO_2
in large foam-polystyrene containers and transported by
express services usually by air. This adds considerably
to their cost. On receipt they are aliquoted and stored
at -20°C. Even at -20°C some have a relatively brief
shelf life and need to be replaced after a few months.
A technique which would enable such enzymes and other
molecular biology reagents to be transported and stored
at room temperature or above with a long shelf life
would be of considerable value.

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We have found that all restriction enzymes, ven the
most unstable, can be dried without loss of activity.
In the dried form these preparations show very
surprising stability and can be kept at up to +55°C for
prolonged periods with no detectable loss of activity.

Other sugars and sugar derivatives in contrast have
no preservative effect on the stability of these
enzymes. Drying in the presence of effective
preservatives such as lactitol enables entirely new
techniques for using restriction enzymes to be
developed. In one such process, the enzymes are dried
in the plastic wells of multi-well format microtitration
plate (e.g. one with 96 wells). For use, the DNA to be
cut is added to the dry well containing the appropriate
enzyme in dried form. The water in which the DNA is
dissolved, acts as a solvent for the dried enzymes which
immediately regain full activity and cut the DNA in a
manner identical with the activity of fresh enzyme.
This simple one step process should be compared with
existing techniques which require that frozen tubes of
enzyme be removed from the freezer, aliquoted on ice,
diluted in special buffers, added to fresh tubes to
which the DNA is subsequently added and then incubated;
a more labour intensive operation. For certain
applications such as Restriction Fragment Length
Polymorphism (RFLP) analysis, a collection of several
enzymes is used to cut the same DNA and the pattern of
fragments produced by each enzyme is analysed by gel

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electrophor sis. This technique can be greatly
simplified by drying the r quisite panel of enzymes in
rows of 96 well plates. Thus up to 8 rows of 12 enzymes
(or 12 rows of 8 enzymes) can be stored in a single
plate. In use, multiple tip pipettes are used to add
DNA simultaneously to a whole row of enzymes and all the
digests are performed simultaneously with only one
manoeuvre.

Dried plates of this type are conveniently stored on
a laboratory shelf and do not require either freezing or
refrigeration. They are entirely stable for very
prolonged periods. Accelerated ageing tests indicate a
shelf life of at least 12 months at room temperature.
It is probable that the life span is essentially
indefinite.

Remarkably, certain sugars which have been claimed
by others to have a preservative function are not useful
and in some cases positively harmful to the preservation
of the biological function of proteins. Thus lactose,
and especially palatinose, accelerate the irreversible
photobleaching of R-phycoerythrin in solution and
completely fail to preserve the activity of restriction
enzymes. Sucrose preserves the biological activity of
restriction enzymes immediately after drying, but on
storage at 37°C progressive damage occurs to the enzymes
and after 5 weeks at this temperature, all activity is

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lost. In contrast trehalose, the disaccharide sugar alcohols lactitol (galactopyranosyl-sorbitol), glucopyranosyl mannitol and glucopyranosyl sorbitol give good preservation of activity. With these sugar alcohols this stabilizing effect is apparently indefinite at elevated temperatures. This is also true of the non-reducing higher sugars such as the trisaccharides raffinose and melezitose. The reducing trisaccharide maltotriose shows no preservative activity on storage at 37°C for 2 weeks. The non-reducing tetrasaccharide stachyose is a good preservative.

In essence, the ability of added carbohydrate to preserve protein stability at elevated temperatures (ambient and above) correlates completely with the absence of a reducing function in the carbohydrate. This can however be an indirect function of the stability of the disaccharide bond. Thus sucrose is a good stabilizing agent short term but within a few weeks at 37°C the protein is destroyed, presumably because of chemical breakdown of the glycosidic bond in sucrose (one of the highest bond energies in biochemistry) which causes secondary chemical damage to the macromolecules.

Absence of a reducing group is not sufficient to guarantee stabilizing ability. Thus the monosaccharide sugar alcohols galactitol, mannitol and erythritol are not satisfactory protective agents. This is not due to

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the fact that a ring structur is required for
stabilizing activity. Sorbitol (glucitol), a straight
chain non-reducing monosaccharide alcohol, does have
some limited activity as a stabilizing agent while
myo-inositol, a non-reducing 6 carbon ring compound is
without stabilizing activity.

Of particular importance is the observation that the
compounds which stabilize proteins during drying also
protect proteins against free radical damage in
solution. While we do not wish to be bound by theory,
we speculate that this effect is not due to the
molecules acting as free radical scavengers because the
effective compounds are chemically the most non-reactive
and therefore most unlikely to compete for free-radical
interaction. It seems more likely that these
carbohydrate molecules are able to bind to proteins in
solution and act as a buffer between the protein and
free radicals produced in the surrounding solvent.
Being much larger than water molecules and therefore
posessing a much higher moment of inertia, it seems
likely that the short lived free radicals cannot
displace hydrogen bonded sugar molecules (and damage the
protein) within their short life-times. The
disaccharide palatinose (isomaltulose) is actively
destructive in this test thus showing that single
examples of a stabilizing effect of a particular sugar
are valueless in predicting its usefulness as a
prot ctiv agent for biological molecules.

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We also believe that the ability of effector molecul s to coat proteins and other biological molecules with an amorphous phase of hydrogen-bonded carbohydrate is an important element in their protective effect. This is exemplified by experiments in which the fluorescent protein phycoerythrin was dried from 0.6M trehalose solution under conditions in which part of the solidifying mixture crystallised and part solidified as an amorphous glass. Only in the latter phase was the fluorescence of RP-E preserved. Similar data was obtained in drying agarose gels. Where trehalose was used as a preservative, excellent preservation of the agarose structure, as shown by the ability of the dried gel to rehydrate to its original volume, was obtained where amorphous glass transformation occured. If the trehalose crystallised during drying, the agarose structure was not preserved and the gels in the area in which crystals formed did not reconstitute on addition of water. When agarose gels were dried in the presence of a sugar derivative which always formed an amorphous glass, such as lactitol, the gels showed uniform and complete preservation of structure and function.

The preservative properties of the non-reducing oligosaccharides and oligosaccharide alcohols were not confined to air drying or high temperature storage, nor to the preservation of proteins and carbohydrates. Lyophilised materials also showed excellent protective

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effects of the sam carbohydrates as detail d above. In
addition, lipids, sp cially lipids dispersed as
 mulsions or liposomes in aqu ous buffers could be dried
from the same carbohydrate solutions with complete
preservation of their properties.

According to the present invention, we thus provide
a method of stabilizing delicate biological materials
such as proteins, lipids, antibodies etc. for long-term
storage under dry conditions, by drying a mutual
solution of the biological material and a stabilizing
carbohydrate selected from non-reducing sugar alcohol
glycosides and non-reducing oligosaccharides having at
least three saccharide groups.

We also provide stabilized biological substances
formed by drying a solution of the substance in the
presence of one of said stabilizing carbohydrates. The
invention can thus be seen as having the same
applicability as our earlier trehalose process.

We also provide stabilized solutions/suspensions in
water.

The following Examples illustrate the invention.

Example 1

The typical restriction endonuclease Pst I is
commonly used for molecular cloning and gives a very

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charact ristic pattern of phage lambda DNA fragment
lengths in electrophoresis gels.

The enzyme was diluted in sterile 96-well U-bottomed
polystyrene or PVC plastic plates. These were air dried
in a laminer flow hood for 12-16 hours at room
temperature (about 18-25°C) and stored at room
temperature, 37°C or 55°C for different periods of time
for accelerated ageing studies. The drying mixture
consisted of three units of the enzyme in 0.45 or 0.6M
trehalose or other sugar or sugar derivative in the
presence of a proprietary buffer at 10μl/well. The
single buffer enabled cutting of DNA with all
restriction enzymes and contained a molecular "water
pump" component (see PCT/GB89/00093).

Buffer: 33mM   Tris acetate buffer (pH7.4)

                     66mM   Sodium acetate

                      1mM   Magnesium acetate

                      1mM   Dithiotreitol

                      3mM   Spermidine

                 0.1mg/ml Bovine Plasma Albumin

Results

The activity of fresh and dried enzyme was
determined by assaying their ability to produce the
characteristic pattern of bands from 0.3 μg of lambda

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bacteriophag DNA dig sted at 37°C for 30 mins and s parat d by l ctrophor sis on 1% agaros gels. Enzymes showed no loss of activity after 10 weeks at 55°C. To confirm full retention of enzymatic activity one experiment was also done in which limiting amounts of enzymes were titrated out from 5 units to 0.5 units per well and used to cut 0.3 µg lambda DNA. Trehalose-dried titrated enzymes were compared with titrations of fresh enzymes. After storage for 2 weeks at 37°C the enzyme titred identically to fresh enzyme showing no loss of activity on drying and storage.

Since it is well known that long term damage to macromolecules dried in sugars can result from chemical attack on the macromolecule by the reducing groups in certain mono and disaccharides, these experiments were repeated with several alternative sugars and their non reducing alcohol derivatives. Pst I was dried as above and also in buffers containing a large number of other sugars and chemical derivatives of common sugars. Trehalose again showed 100% preservation (Table 1a and b).

Table 1a: Storage of dried Pst I in Different Carbohydrates

CARBOHYDRATE	CHEMICAL NAME	RED	TEMP	TIME	PRESERVATION
Glucose	α-D-glucopyranose	+	37°	1	+
			=	14	-
Galactose	α-D-galactopyranose	+	=	1	-
Mannose	α-D-mannopyranose	+	=	1	-
Sorbitol	D-glucitol	-	=	1	++
			=	14	+
Galactitol	6 carbon alcohol	-	=	70	-
Mannitol	"	-	=	1	-
Erythritol	4 carbon alcohol	-	=	14	-
Myo-Inositol	(1α,2α,3α,4β,5α,6β)cyclo-hexanehexol	-	=	14	-
Threitol	4 carbon alcohol	-	=	14	-
Trehalose	α-D-glucopyranosyl-α-D-glucopyranoside	-	=	14	+++
			=	70	+++
Sucrose	β-D-fructofuranosyl-α-D-glucopyranoside	-	55°	70	+++
Maltose	4-O-α-D-glucopyranosyl-D-glucose	+	37°	14	++±
			=	35	-
Lactose	4-O-β-D-galactopyranosyl-glucopyranose	+	=	14	-
Palatinose	α-D-glucopyranosyl-1,6-fructose	+	=	14	-
Lactulose	4-O-β-D-galactopyranosyl-D-fructose	-	=	14	-
Maltitol	4-O-β-D-glucopyranosyl-D-glucitol	-	=	14	+++
			=	70	+++
Lactitol	4-O-β-D-galactopyranosyl-D-glucitol	-	55°	14	+++
				70	+++
				70	+++

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Table 1b: Storage of Dried Pst I in Various Carbohydrates

CARBOHYDRATE	CHEMICAL NAME	RED	TEMP	TIME	PRESERVATION
Palatinit	1:1 mixture glucopyranosyl-1→6-mannitol+1,6-sorbitol	-	37°	14 70	+++ +
Palatinit isomer 1	α-D-glucopyranosyl-1→6-mannitol	-	37°	14 70	+++ +
Palatinit isomer 2	α-D-glucopyranosyl-1→6-sorbitol	+	"	14 70	+++ +++
Maltotriose	O-α-D-glucopyranosyl(1→4)-O-α-D-glucopyranosyl(1→4)-D-glucose	-	"	14	-
Raffinose	β-D-fructofuranosyl-O-α-D-galactopyranosyl-(1→6)-α-glucopyranoside	-	"	14	+++
Melezitose	O-α-D-glucopyranosyl-(1→3)-β-D-fructofuranosyl-α-D-glucopyranoside	-	"	14	+++
Stachyose	β-D-fructofuranosyl-O-α-D-D-galactopyranosyl-(1→6)-β-D-fructofuranosyl-α-D-glucopyranoside	-	"	14	+++
Methyl glucoside	methyl-α-D-glucopyranoside	-	"	14	-
Methyl mannoside	methyl-α-D-mannopyranoside	-	"	14	-
Lactulose		+	"	14 35	+ -

RED = reducing sugar

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Neith r palatinose nor lactose preserved any
enzymatic activity. When lactitol was substituted for
palatinose or lactose, activity was preserved at all
temperatures in a manner equivalent to trehalose i.e.
all enzymes were fully active after 10 weeks at 55°C.

Sugar alcohols of the reducing disaccharides gave
much better results than the parent disaccharide
suggesting that complete lack of reducing activity plus
lower-energy glycosidic linkages which resist chemical
attack are an important feature of trehalose and other
potential preservatives. Interestingly, sugar alcohols
of the monosaccharides were poor preservatives except
for sorbitol which was only modestly effective. With
the higher sugars, again only the non-reducing
trisaccharides and the non-reducing tetrasaccharide
stachyose were effective.

Example 2

The fluorescent phycobiliprotein R-phycoerythrin is
a 240 KD protein extracted from red algae. It has a
fluorescence efficiency of 0.98 and emits orange-red
light when illuminated with 490 nm blue light. The
molecule is very susceptible to desiccation damage and
cannot even be lyophilised without collapse of molecular

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structur and loss of fluor sc nc . R-PE is also
d natur d in solution by excitation light in the UV-Blue
band of the spectrum. This denaturation is mediated by
free radicals created in the solvent water by the
irradiation. Free radical damage is also reduced in a
dose dependent manner by added trehalose and certain
other sugars and sugar alcohols but is, unexpectedly,
greatly accelerated by other sugars such as palatinose
(Table II)

Table II

Carbohydrate	% loss of fluorescence of R-PE solutions	
	5 min	20 min
trehalose	9%	48%
isomaltulose	93%	100%
lactitol	13%	47%
glucopyranosyl-mannitol (GPM)	21%	47%
glucopyranosyl-sorbitol (GPS)	7%	43%
nil	17%	55%

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From th se results it will b s en that whil the
tw r duc d forms of isomaltulose are eff ctiv in
reducing the loss of fluorescence, isomaltulose itself
exhibited a disastrous effect on the fluorescence.

The same panel of sugars and derivatives was used to
dry and store a variety of other biological molecules at
various temperatures, with essentially similar results.

Example 3

An immunoconjugate produced by covalent bonding of
the $F(ab)_2$ fragment of rabbit antibodies against rat
immunoglobulin to peroxidase obtained from horseradish.

The conjugate was dried in various sugars and
alcohols at a concentration of 15 migrogram per ml and
10% sugar by weight and stored for 2 or 4 weeks at 37°
or 55°C and titrated for its activity on plates coated
with a rat monoclonal antibody.

In trehalose no activity was lost at all. The
average values for other sugars and alcohols at 4 weeks
were:

GPM	= 86%	; maltitol	67%;	maltose	49%
lactitol	= 86%	; palatinit	68%;	GPS	57%
glucose	= 12%	; no sugar	0%;		

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Exampl 4

    Agarose gels 7.5 cm x 10 cm x 0.6 cm were poured from molten agarose (1% w/v) containing 15% by weight of various sugars. The gels were dried and stored at various temperatures. When rehydrated, the % regain of vel volume was measured and the gels were used in electrophotosis to separate the Hind III fragments of phage Lambda DNA as a check on function.
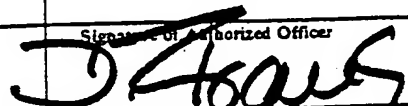

Results

    All gels failed to rehydrate and to separate DNA after storage at 55°C which seems to be above the re-melting temperature of agarose. At 37°C lactitol and trehalose were perfect from > 12 weeks. Also perfect were GPs and GPM. Palatinose failed after 2 weeks, glucose, mannitol and sorbitol failed after 1 week.

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CLAIMS:-

1.   A method of preserving delicate biological
substances or organic compounds (a) in a dry state
and/or (b) at elevated temperatures and/or (c) under
irradiation comprising incorporating in a system
containing the said substances or compounds a sugar or a
sugar derivative selected from a (i) non-reducing
glycoside of a polyhydroxy compound selected from sugar
alcohols and other straight chain polyalcohols, or
(ii) a non-reducing oligosaccharide selected from
raffinose, stachyose and melezitoze.

2.   A method according to claim 1 wherein the glycoside
is a monoglycoside.

3.   A method according to claim 2 wherein the glycoside
is obtained by reduction of disaccharide.

4.   A method according to claim 1 wherein the glycoside
comprises a glucoside or galactoside.

5.   A method according to claim 4 when the glycosideis a
glycoside of sorbitol or mannitol.

6.   A method according to claim 1 wherein the glycoside
comprises maltitol; lactitol or one or both isomers of
palatinit, the sugar alcohol derived from isomaltulose.

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CORRECTED VERSION

INTERNATIONAL SEARCH REPORT

International Application No **PCT/GB 91/00759**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]
According to International Patent Classification (IPC) or to both National Classification and IPC
Int.Cl.5   C 12 N 9/96   A 61 K 47/26

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]		
Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Patent Abstracts of Japan, C-Field, 17 April 1987, & JP, A, 61260882 (WAKAMOTO PHARMACEUT CO. LTD) 19 November 1986, see abstract ---	1-6
X	Patent Abstracts of Japan, 13 November 1986, & JP, A, 61139384 (NIPPON SHINYAKU CO. LTD) 26 June 1986 ---	1-6
X	Chemical Abstracts, vol. 95, 1981, (Columbus, Ohio, US), Ooizumi Tooru et al.: "Quantitative aspect for protective effect of sugar and sugar alcohol against denaturation of fish myofibrils", see page 517, abstract no. 185699n, & Nippon Suisan Gakkaishi 1981, 47(7), 901-8 --- -/-	1-6

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION	
Date of the Actual Completion of the International Search 19-07-1991	Date of Mailing of this International Search Report 0 9. 09. 91
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer Mme Dagmar FRAN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Chemical Abstracts, vol. 110, 1989, (Columbus, Ohio, US), K. Gekko et al.: "Amino acid solubility and protein stability in aqueous maltitol solutions", see page 292, abstract 110409n, & Agric. Biol. Chem. 1989, 53(1), 89-95 ---	1-6
X	FR,M, 7773 (INSTITUT MERIEUX) 23 March 1970, see page 1, line 36 - page 2, line 6; claim 1 ---	1
X	Japanese Patents Gazette, week 8538, 1985, no. J60149-972A (IATRON LABORATORIES), see abstract ---	1
X	Japanese Patents Gazette, week 8825, 1988, no. J63106-562A (GREEN CROSS), see abstract -----	1

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ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

GB 9100759
SA 47465

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 15/10/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-M- 7773	23-03-70	None	

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